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Evaluation of rice (*Oryza sativa* L.) germplasm for biotic and abiotic stresses and their genetic diversity using SSR markers

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ABSTRACT

The present study was carried out to screen the rice germplasm for biotic and abiotic stress tolerance. Fifty genotypes were screened using 18 microsatellite marker pairs distributed throughout the genome. Blast resistance alleles were observed in the genotypes viz., SYE -8, IRBLSH - KU (CO), TN1, Pusa Sugandha, B40, IPN - 12, IRBLZ - FU/RL, IR09L324 and IR10L139. Brown plant hopper resistance genotype includes; IRBLB - ITB (CO), IRBLK - KA (CO), IRBLKS - CO (CO), IRBLTA - ME (CO), IRBLSH - KU (CO), IR09L204, IR10A314 and IR10F221. Bacterial Blight resistant genotypes were; Basmati 370, SYE - 6, SYE - 7, SYE - 75, IRBLK - KA (CO) IRBLKS - CO (CO), IRBLSH - S (CO), IRBLT - K59, IRBLZ5 - CA (CO) and IRBLTA - 2 IR 64 (CO). Gall midge resistant genotypes are CT 186 14-4-1-2-3-2, SYE-4, SYE-5, SYE-6, IR09N251, IR09N516 and IR10A237 observed in the present studies. Four genotypes viz., IR10A237, IR10A314, IR10F221 and IR10A237 were observed tolerant to drought condition, since it showed amplification at specific base pair. Salt tolerant genotypes include; Ajaya, Durai, SYE-4, SYE-7, SYE-8, IRBLK-KU (CO), IRBLKP-K60, IRBLKS-CO (CO), IRBLTA-ME(CO), IR09N501, IR09N516, Basmati 370, TN1, Pusa Sugandha, B40, IPN-12, K90San, CT 186 14-4-1-2-3-2, IRBLSH-B(CO), IRBLSH-S(CO), IR05A272, IR09L324, IR09N251 and IR10A231. Out of total 18 SSR primers used in this study, 14 primers amplified and showed the polymorphism with357 loci. Each primer thus produced on an average 25.5 loci in the size ranging from 164.28 bp to 292.85 bp in the 50 rice genotypes in relation to diversity assessment. UPGMA grouped 50 rice genotypes into two main clusters, which were further divided into two sub-clusters. This study will be helpful for selection of parental lines and development of new breeding population tolerant to specific traits through marker assisted selection.

Key words: *Abiotic and biotic stress, germplasm, MAS, marker, polymorphism, SSR*

INTRODUCTION

Rice, *Oryza sativa* L. (2n=24) belonging to the family Graminae; subfamily Oryzoidea is the staple food for one third of the world's population. It occupies almost one-fifth of the total area covered under cereals. It has been cultivated in India and China for several thousands of years (Poehlman and Sleper, 1995). During the past decade, phenotypic characteristics have been used for classification and identification of species or varieties. Taking into account the utility, the conservation of genetic diversity and building up of nuclear base populations are essential for the improvement of cereal crops. Molecular markers have provided a powerful new tool for breeders to search for new sources of variation and to investigate genetic factors controlling quantitatively inherited traits (Terzi et al., 2005). The wide array of DNA markers are available, microsatellite or Simple Sequence Repeat (SSR) markers are considered to be appropriate for assessment of genetic diversity and variety identification because of their ability to detect large numbers of

discrete alleles repeatedly, accurately and efficiently (Smith and Helentjaris, 1996). SSR markers have been extensively used to analyze genetic structure within the cultivated rice (Garris et al., 2005) and to evaluate genetic diversity among strains of wild rice and among cultivars of cultivated rice (Ren et al., 2003; Yu et al., 2003). Genetic diversity is normally assessed by common morphological traits. However, such traits are affected by effects of environment, development stage of the plant and the type of plant material. Therefore, it requires several replications to establish the genotypic contributions (Prabhu and Ganesan, 2013). The marker-assisted selection (MAS) of resistance genes will help in the breeding program to develop multi-stress resistant rice varieties. Hence, there is a need to go for a highly reliable and precise method for assessment of genetic variability with no environmental effects.

Rice crop is prone to biotic and abiotic stresses. Biotic stresses include insect pests, fungus and bacteria. Among abiotic stresses, drought, cold, and salinity are well studied in rice and it has been suggested that they reduce average yields by >50 percent (Bray et al., 2000). The emergence of new diseases and pests and the changing climate are the major issues that address the requirement for sustainable crop development and resistance to biotic and abiotic stresses (Hasan et al., 2015). Various genes have been identified, cloned, and characterized to combat these stresses and protect rice crops. Genetic improvement of rice for fungus resistance is the need of the day due to vulnerability of this crop to various pathogens, including fungi, bacteria, and viruses as well as abiotic stresses including drought, high and low temperatures, salinity, submergence, and oxidative stress. Thus keeping eye on thirst of the time, the present investigation for valuation of Rice germplasm for biotic and abiotic stresses through SSR markers was undertaken.

MATERIALS AND METHODS

Plant material and DNA extraction

The experimental material consisted of 50 rice germplasm that were obtained from Agricultural Research Station Karjat, Dist. Raigad, Maharashtra, India. Details of germplasm are given in Table 1. Genomic DNA was extracted from young leaf tissues using the protocol of Edwards (1991) *i.e.*, rapid method with some modifications. Purification of DNA in some sample was done to remove RNA and proteins, which were the major contaminants. RNA was removed by adding RNase to the DNA sample @100 µg ml^{-1} and incubated at 37°C for 1 hour. The quality of DNA in the sample was determined after agarose gel electrophoresis with standard DNA *i.e.*, uncut lambda Hind III DNA on 1% agarose gel.

Table 1. List of rice germplasm.

Sr.No	Germplasm	Sr.No	Germplasm
1	Ajaya	26	IRBLSH-S (CO)
2	Durai	27	IRBLT-K59
3	Basmati-370	28	IRBLZ5-CA (CO)
4	TN1	29	IRBLZ-FU/RL
5	PusaSugandha	30	IRBLTA-2 IR64 (CO)
6	B40	31	IR1552
7	IPN-12	32	IRO4A216
8	K 90 San	33	IRO5A272
9	CT 186 14-4-1-2-3-2	34	IRO6A145
10	SYE -1	35	IRO8L216
11	SYE-4	36	IRO9L204
12	SYE-5	37	IRO9L324
13	SYE-6	38	IRO9N127
14	SYE-7	39	IR09N251
15	SYE-8	40	IRO9N501
16	SYE-75	41	IRO9N516
17	IRBLB-ITB[CO]	42	IR10A121
18	IRBLK-KA[CO]	43	IR10A199
19	IRBLK-KU (CO)	44	IR10A231
20	IRBLKM-T5(CO)	45	IR10A237
21	IRBLKP-K60(CO)	46	IR10A314
22	IRBLKS-CO (CO)	47	IR10F221
23	IRBLTA-ME(CO)	48	IR10L139
24	IRBLSH-B (CO)	49	IR10L185
25	IRBLSH-KU (CO)	50	PhuleSamrudhi

Microsatellite analysis

For the molecular screening of rice germplasm, 18 different trait specific Simple Sequence Repeat (SSR) markers well distributed on all the 12 chromosomes of rice were used (Table 2). These SSR markers were chosen based on their physical position on the 12 chromosomes of rice genome according to the 'Gramene' database (http//:www.gramene.org). PCR was performed in 96-well plates containing a total of 20 µl volume; 35 ng DNA template, 10X PCR buffer, 15 mM $MgCl_2$, 10 mMdNTPs, 5 PMol forward and reverse primers, 3 U Taq polymerase enzyme and nano-pure water. PCR amplified products were separated by 2.5% Agarose gel electrophoresis.

Table 2. List of SSR primers with their sequences and linked gene.

Sr.No	Primer	Sequence Forward primer	Sequence Reverse Primer	Amplicon Size (bp)	Annealing Temperature(ºC)	Linked gene
1.	RM 140	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG	244-329	62.1	*Saltol*
2.	RM 1287	CCATTTGCAGTATGAACCATGC	ATCATGCAATAGCCGGTAGAGG	158-207	56.7	*Saltol*
3.	RM 562	GGAAAGGAAGAATCAGACACAGAGC	GTACCGTTCCTTTCGTCACTTCC	126	62.5	*Saltol*
4.	RM 6775	AATTGATGCAGGTTCAGCAAGC	GGAAATGTGGTTGAGAGTTGAGAGC	192	58.7	*Bph25*
5.	RM 5479	CTCACCATAGCAATCTCCTGTGC	ACTTCGTTCACTTGCATCATGG	152	60.0	*Bph26*
6.	RM-5926	ATATACTGTAGGTCCATCCA	AGATAGTATAGCGTAGCAGC	135	58.0	*Pi1*
7.	RM 8225	GCGTGTTCAGAAATTAGGATACGG	GATCTCGCCACGTAATTGTTGC	221	58.7	*Pi*
8.	RM 206	ATCGATCCGTATGGGTTCTAGC	GTCCATGTAGCCAATCTTATGTGG	140	58.1	*Pi54*
9.	RM 212	AAGGTCAAGGAAACAGGGACTGG	AGCCACGAATTCCACTTTCAGC	135	62.9	*Dr*
10.	RM 302	TGCAGGTAGAAACTTGAAGC	AGTGGATGTTAGGTGTAACAGG	135-140	61.7	*Dr*
11.	RM 3825	CCACTAGCAGATGATCACAGACG	GAGCACCTCATAAGGGTTTCAGC	147	62.8	*Dr*
12.	RM 122	CTTCTTCCGCTTCCTCCTTCC	TGTACCAGTGCACCGAGAGTTGG	250	61.5	*Xa13*
13.	RM 22709	CGCGTGGGCGAGACTAATCG	CCTTGACTCCGAGGATTCATTGTCC	170	65.9	*Gm8*
14.	RM 547	TTGTCAAGATCATCCTCGTAGC	GTCATTCTGCAACCTGAGATCC	270	56.1	*Gm4*
15.	RM-8094	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA	171-280	58.0	*Saltol*
16.	RM 309	CACGCACCTTTCTGGCTTTCAGC	AGCAACCTCCGACGGGAGAAGG	152	66.9	*Bph26*
17.	RM 1233	ATGGGCACGTGTAATTCATTCG	ATCCTCGAAAGTAGGAGTAGGAAAG	160	56.1	*Xa4*
18.	pTA248	AGACGCGGAAGGGTGGTTCCCGGA	AGACCGGGTAATCGAAAGATGAAA	900-1000	54.0	*Xa21*

Data analysis

The images of gels were carefully studied and amplicons, which occurred only once for particular genotypes were, marked which constituted the band for that particular genotype. Additionally, the band fragments present in two genotypes were also marked, which in combination with other bands generated with other primers would constitute the screenings. Molecular size of each amplified fragments were analyzed by using Uvi Tech, Fire reader software programme. Markers were scored for the presence (1) or absence (0) of the corresponding band among the genotypes. UPGMA cluster analysis was performed using Jaccard's similarity coefficient matrices calculated from SSR markers to generate a dendrogram across 55 rice varieties. A pair wise similarity index (SI) was calculated and the UPGMA based dendrogram of 55 rice varieties generated with Multivariate Statistical Package (MVSP).

RESULTS AND DISCUSSION

Molecular markers play an important role and are an extremely powerful tool for varietal identification and determining variability between genetic materials of the cultivars. Various genes have been identified, cloned, and characterized to combat these stresses and protect rice crops. Due to the proliferation of rice varieties with a narrow genetic base, the morphological traits and biochemical markers to discriminate rice are not adequate.

Blast resistance

Rice varieties with number of alleles in common for any specific resistance might have a similar blast R gene, and understanding the natural diversity at the specific gene is important for incorporation of specific R gene using DNA marker into rice breeding program. Out of two primers that were used to identify blast resistance allele linked in 50 rice varieties, RM-8225 conferred resistance at 221bp in two varieties *viz.*, SYE - 8 and IRBLSH - KU (CO) (Fig. 2). RM5926 shows resistance link allele at 135bp in eight varieties *viz.*, IRBLSH - KU (CO), TN1, Pusa Sugandha, B40, IPN - 12, IRBLZ - FU/RL, IR09L324 and IR10L139 (Table 5). Results of the present study are in agreement with the reports on blast resistance by Fjellstrom et al. (2004), Roychowdhury et al. (2012) and Anupam et al. (2017).

Table 3. Molecular polymorphism, PIC Values, no. of alleles and size of loci revealed by SSR primers in rice genotypes.

Sr. No.	Primer	Total no. of polymorphic band	% Polymorphism	No. of alleles	PIC	Range of amplified products
1	RM 140	50	100	26	0.34	200-300
2	RM 1287	50	100	25	0.42	100-250
3	RM 562	50	100	22	0.42	100-200
4	RM 6775	50	100	20	0.14	150-250
5	RM 5479	51	100	23	0.39	250-350
6	RM-5926	50	100	26	0.48	100-200
7	RM 8225	50	100	29	0.54	200-350
8	RM 206	50	100	20	0.28	350-500
9	RM 212	50	100	27	0.36	100-250
10	RM 302	63	100	29	0.63	150-350
11	RM3825	50	100	22	0.50	150-250
12	RM 122	48	100	29	0.41	200-300
13	RM 22709	50	100	23	0.32	100-150
14	RM 547	76	100	36	0.61	150-400
Total		738	-	357	-	-
Average		52.71	100.00	25.5	0.41	164.28-292.85

Brown plant hopper resistance

Appropriate evaluation of germplasm for BPH resistance is the key to study the genetics of BPH resistance and to identifying genuine resistance genes. The eight rice varieties *viz.*, IRBLB - ITB (CO), IRBLK-KA (CO), IRBLKS-CO (CO), IRBLTA-ME (CO), IRBLSH-KU(CO), IR09L204, IR10A314 and IR10F221 were identified to be carrying resistant linked allele to BPH using SSR markers *viz.*, RM5479, RM6775 and RM6775 marker which confers resistance at 192bp, which is located on chromosome 6, for pathotype Bph25 (Table 5). The tracking of the genes for resistance is possible by following the path of markers that are linked to each gene for resistance. Similar results were obtained using molecular markers on BPH resistance by Myint et al. (2012), Shabanimofrad et al. (2015) and Bhogadhi et al. (2015).

BLB-resistance

Till date, approximately 34 genes (23 dominant and 11 recessive) conferring resistance against various strains of *X. oryzae*, have been identified. Major resistance genes, including *Xa4*, *Xa5*, *Xa7*, *Xa13* and *Xa21* have been incorporated into rice cultivars, in order to develop new resistant. To determine the resistance and susceptibility status for BLB, RM122 linked marker to *Xa13* (250bp) exhibited different levels of polymorphism. RM122 (*Xa13*) showed the highest level of polymorphism in varieties like Basmati 370, SYE-6, SYE-7, SYE-75, IRBLK-KA(CO), IRBLKS-CO(CO), IRBLSH-S(CO), IRBLT - K59, IRBLZ5-CA(CO) and IRBLTA-2, IR-64(CO) (Table 5).

Gall midge resistance

The flanking SSR markers RM547 and RM22709 were used and reported the presence of resistance linked alleles for biotypes *Gm4* (270bp) and *Gm8* (160 to 170bp), respectively. The varieties CT 186 14-4-1-2-3-

Table 4. Clustering pattern of 50 rice genotypes.

Cluster		No. of genotypes	Name of the genotypes
I	IA	14	IR10A231, IR10A121, IRO9N501, IRO9N516, IRO9L324, IRO9N127, IRO9L204, IR09N251, IRO8L216, IR10A199, IRO5A272, IRO6A145, IR1552, IRO4A216.
	IB	7	IR10L139, IR10F221, PhuleSamrudhi, IR10L185, IR10A314, IR10A237, IRBLZ-FU/RL.
II	IIA	6	IRBLSH-KU (CO), IRBLTA-ME(CO), IRBLSH-B (CO), IRBLKS-CO (CO), IRBLKP-K60(CO), IRBLKM-T5(CO).
	IIB	23	IRBLZ5-CA (CO), SYE-7, SYE-4, IRBLTA-2 IR64 (CO), IRBLT-K59, IRBLSH-S (CO), IRBLB-ITB[CO], SYE-75, SYE-8, SYE -1, SYE-6, SYE-5, B40, IPN-12, PusaSugandha, Basmati 370, Durai, IRBLK-KU (CO), IRBLK-KA[CO], CT 186 14-4-1-2-3-2, K 90 San, TN1, Ajaya.

Table 5. Germplasms shows multiple resistance / tolerance for different traits.

Sr.No	Name of Genotype	Blast	BPH	Blight	Gall midge	Drought	Salt
1	Ajaya	-	-	-	-	-	+
2	Durai	-	-	-	-	-	+
3	Basmati-370	-	-	+	-	-	+
4	TN1	+	-	-	-	-	+
5	PusaSugandha	+	-	-	-	-	+
6	B40	+	-	-	-	-	+
7	IPN-12	+	-	-	-	-	+
8	K 90 San	-	-	-	-	-	+
9	CT 186 14-4-1-2-3-2	-	-	-	+	-	+
10	SYE -1	-	-	-	-	-	-
11	SYE-4	-	-	-	+	-	+
12	SYE-5	-	-	-	+	-	-
13	SYE-6	-	-	+	+	-	-
14	SYE-7	-	-	+	-	-	+
15	SYE-8	+	-	-	-	-	+
16	SYE-75	-	-	+	-	-	-
17	IRBLB-ITB[CO]	-	+	-	-	-	-
18	IRBLK-KA[CO]	-	+	+	-	-	-
19	IRBLK-KU (CO)	-	-	-	-	-	+
20	IRBLKM-T5(CO)	-	-	-	-	-	-
21	IRBLKP-K60(CO)	-	-	-	-	-	+
22	IRBLKS-CO (CO)	-	+	+	-	-	+
23	IRBLTA-ME(CO)	-	+	-	-	-	+
24	IRBLSH-B (CO)	-	-	-	-	-	+
25	IRBLSH-KU (CO)	+	+	-	-	-	-
26	IRBLSH-S (CO)	-	-	+	-	-	+
27	IRBLT-K59	-	-	+	-	-	-
28	IRBLZ5-CA (CO)	-	-	+	-	-	-
29	IRBLZ-FU/RL	+	-	-	-	-	-
30	IRBLTA-2IR64(CO)	-	-	+	-	-	-
31	IR1552	-	-	-	-	-	-
32	IR04A216	-	-	-	-	-	-
33	IR05A272	-	-	-	-	-	+
34	IR06A145	-	-	-	-	-	-
35	IR08L216	-	-	-	-	-	-
36	IR09L204	-	+	-	-	-	-
37	IR09L324	+	-	-	-	-	+
38	IR09N127	-	-	-	-	-	-
39	IR09N251	-	-	-	+	-	+
40	IR09N501	-	-	-	-	-	+
41	IR09N516	-	-	-	+	-	+
42	IR10A121	-	-	-	-	-	-
43	IR10A199	-	-	-	-	-	-
44	IR10A231	-	-	-	-	-	+
45	IR10A237	-	-	-	+	+	-

2, SYE - 4, SYE - 5, SYE - 6, IR09N251, IR09N516 and IR10A237 were identified to be carrying resistant linked allele through this study. Various studies done by Bentur et al. (2011), Sama et al. (2012) and Kumar et al. (2013), revealed that strong association of studied locus specific makers and these are in agreement with our results (Table 5).

Drought tolerance

The current challenge is to decipher the complexities of drought resistance in rice and exploit all available genetic resources to produce rice varieties combining drought adaptation with high yield potential, quality, and resistance to biotic stresses. Three SSR markers *viz.*, RM212, RM302 and RM3825 are located on

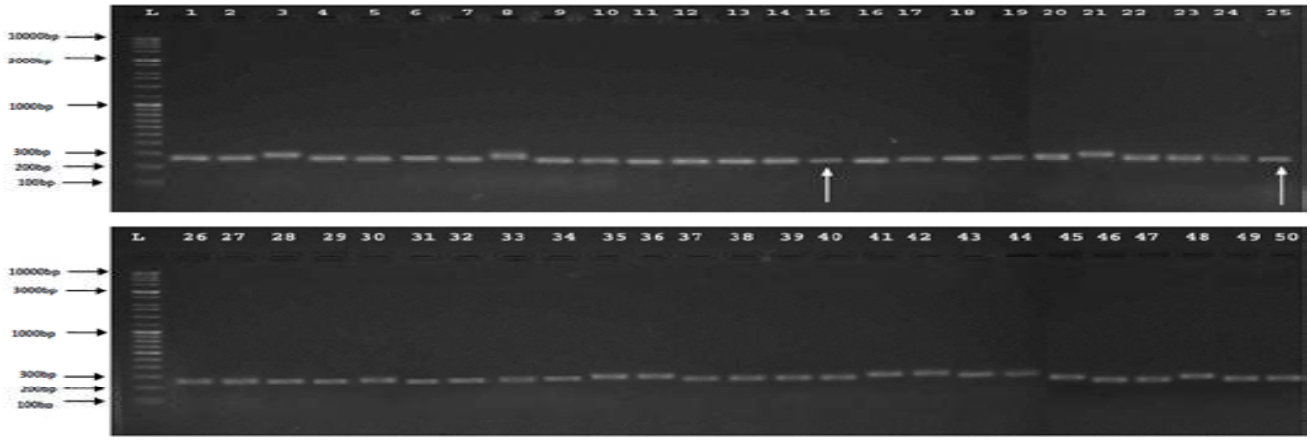

Fig. 1. Amplification of primer RM-8225 specific for blast resistance on agarose gel. L= High range ladder. (Arrow indicates presence of the resistance linked allele.)

chromosome 1 of rice between 135, 135-140 and 147 cM, respectively. Resistance linked alleles *Dr* (135 bp) for the marker RM212 was found to be present in four genotypes IR10A237, IR10A314, IR10F221 and IR10A237 and observed to be tolerant to drought condition, since it showed amplification at specific base pair (Table 5).

Salt tolerance

Salt stress is one of the serious abiotic stresses along with other abiotic stresses in rainfed and lowland areas and in coastal belts of the country. Four markers, *viz*., RM562, RM1287 and RM140 were reported to be located on chromosome 1 of rice between 126, 162 and 248-264 cM, respectively. Based on salt tolerant allele *saltol* linked to RM140 (244-264 bp)was observed in twenty genotypes, followed by 11 genotypes were found have linkage with RM562 (Table 5). These reports are in concurrence with Zeng et al. (2004), Mohammadi-Nijad et al. (2008), Linh et al. (2012), Islam et al. (2012), Al-Amin et al. (2013), Chattopadhyay et al. (2014) and Rubel et al. (2014).

Genetic variability and polymorphism

The polymorphism percentage for individual primer was calculated by the ratio of number of polymorphic bands obtained over the total number of bands produced across the 50 rice germplasm. A total of 18 SSR primer pairs distributed across the genome were used for molecular analysis. Out of that, the 14 microsatellite markers used were found to be polymorphic and showed 100 percent polymorphism. The PIC values of primers ranged from 0.14 in SSR primer RM6775 to 0.63 in SSR primer

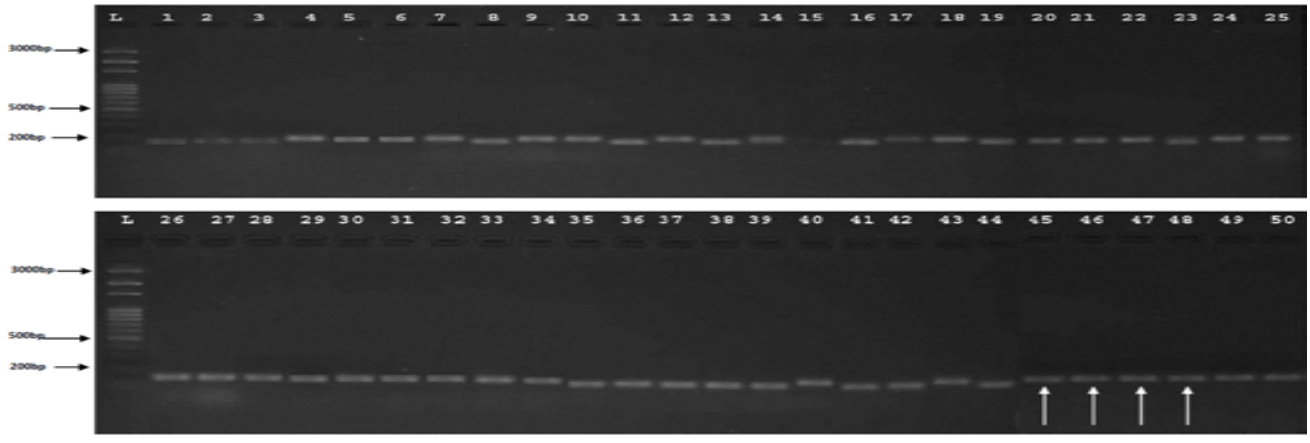

Fig. 2. Amplification of primer RM-212 specific for drought resistance on agarose gel. L=100bp ladder. (Arrow indicates resistance linked allele.)

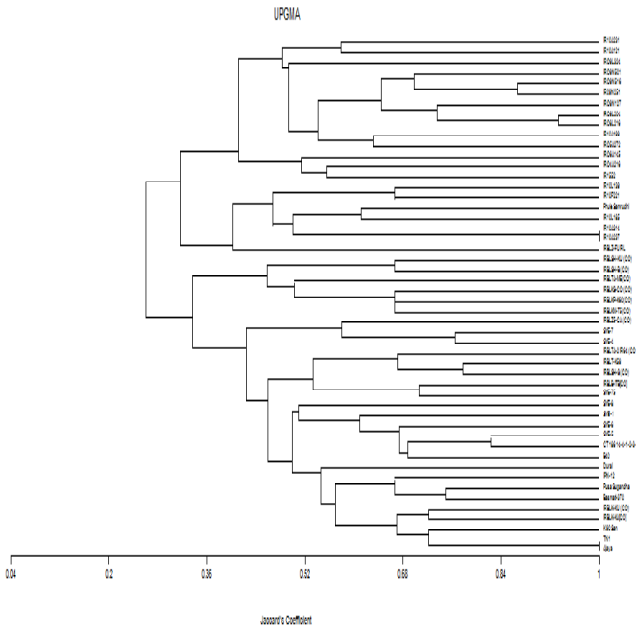

Fig. 3. Dendrogram constructed using Jaccard's similarity coefficient.

RM302 with an average PIC value of 0.41. Further, it was observed that, there was no correlation between per cent polymorphism and PIC values as SSR primers RM6775 showed minimum PIC value but were 100 per cent polymorphic. The higher the PIC value, the more informative is the SSR marker. Hence, primers RM547 and RM302 were found to be highly informative. The pairwise similarity values ranged from 0.038 to1.00. Maximum similarity value of 1.00 was noticed between IR10A314 & IR10A237 as well as and TN1 and Ajaya. Minimum similarity value of 0.038 was observed between IR09N501 and Pusa Sugandha (Table 3). Cluster analysis separated the two major clusters and each cluster consists of two sub clusters (Table 5 and Fig. 4). Therefore, in future, these genotypes can be used directly for cultivation under changing climatic condition and helpful in selection of parents for hybridization program specific to biotic or abiotic stresses. This will help the breeders to develop strategic breeding programs in order to produce elite lines and the availability of fingerprint would be helpful to protect those genotypes from unsolicited commercial exploitation.

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